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Robert G. Collins

Rizwan Velji

Natalia V. Guevara

M. John Hicks

Lawrence Chan

See next page for additional authors

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Robert G. Collins, et. al., (2000) P-selectin or intercellular adhesion molecule (ICAM)-1 deficiency substantially protects against atherosclerosis in apolipoprotein E-deficient mice. *Journal of Experimental Medicine* 191:1189. DOI: <http://doi.org/10.1084/jem.191.1.189>

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Authors

Robert G. Collins, Rizwan Velji, Natalia V. Guevara, M. John Hicks, Lawrence Chan, and Arthur L. Beaudet

P-Selectin or Intercellular Adhesion Molecule (ICAM)-1 Deficiency Substantially Protects against Atherosclerosis in Apolipoprotein E-deficient Mice

By Robert G. Collins,* Rizwan Velji,* Natalia V. Guevara,‡
M. John Hicks,§ Lawrence Chan,‡|| and Arthur L. Beaudet*‡

From the *Department of Molecular and Human Genetics, ‡Department of Cell Biology, §Department of Pathology, and ||Department of Medicine, Baylor College of Medicine, Houston, Texas 77030

Abstract

The expression of leukocyte and endothelial cell adhesion molecules (CAMs) is essential for the emigration of leukocytes during an inflammatory response. The importance of the inflammatory response in the development of atherosclerosis is indicated by the increased expression of adhesion molecules, proinflammatory cytokines, and growth factors in lesions and lesion-prone areas and by protection in mice deficient in various aspects of the inflammatory response. We have quantitated the effect of deficiency for intercellular adhesion molecule (ICAM)-1, P-selectin, or E-selectin on atherosclerotic lesion formation at 20 wk of age in apolipoprotein (apo) E^{-/-} (deficient) mice fed a normal chow diet. All mice were apo E^{-/-} and CAM^{+/+} or CAM^{-/-} littermates, and no differences were found in body weight or cholesterol levels among the various genotypes during the study. ICAM-1^{-/-} mice had significantly less lesion area than their ICAM-1^{+/+} littermates: 4.08 ± 0.70 mm² for -/- males vs. 5.87 ± 0.66 mm² for +/+ males, and 3.95 ± 0.65 mm² for -/- females vs. 5.59 ± 1.131 mm² for +/+ females, combined *P* < 0.0001. An even greater reduction in lesion area was observed in P-selectin^{-/-} mice: 3.06 ± 1.04 mm² for -/- males vs. 5.09 ± 1.22 mm² for +/+ males, and 2.85 ± 1.26 mm² for -/- females compared with 5.60 ± 1.19 mm² for +/+ females, combined *P* < 0.001. The reduction in lesion area for the E-selectin null mice, although less than that seen for ICAM-1 or P-selectin, was still significant (4.54 ± 2.14 mm² for -/- males vs. 5.92 ± 0.63 mm² for +/+ males, and 4.38 ± 0.85 mm² for -/- females compared with 5.94 ± 1.44 mm² for +/+ females, combined *P* < 0.01). These results, coupled with the closely controlled genetics of this study, indicate that reductions in the expression of P-selectin, ICAM-1, or E-selectin provide direct protection from atherosclerotic lesion formation in this model.

Key words: E-selectin • cell adhesion molecules • aorta • cholesterol • intercellular adhesion molecule-1

Introduction

The development of atherosclerosis is influenced by many genetic and environmental factors. These include diet, smoking, and variations in lipid metabolism genes (1). Evidence also suggests a role for the inflammatory response in the pathogenesis of atherosclerosis with the adhesion of circulating leukocytes, especially monocytes, to the endothelium at sites of injury (2, 3). Leukocyte adhesion and emigration into the subendothelial space, in response to chemoattractants and other activating molecules, is mediated by leukocyte and endothelial cell adhesion molecules (CAMs). After migration

into particular lesion-prone areas of the arterial vasculature, some monocytes ingest lipids and become foam cells, initiating a complex chain of events ending in lesion development (2, 3).

Leukocyte emigration from the vasculature occurs in several steps regulated by distinct adhesion molecules. Leukocytes first undergo E-, L-, and P-selectin-mediated rolling along the endothelial surface, followed by firm attachment involving the β₁- and β₂-integrins and Ig adhesion superfamily members such as intercellular adhesion molecule (ICAM)-1 and vascular (V)CAM-1 (4). Several of these molecules, including ICAM-1, VCAM-1, E-selectin, and P-selectin, show increased expression in atherosclerotic lesions (5–9). This includes studies in mice, rabbits, and humans and also includes evidence that a high-fat diet increases expression of various CAMs.

Address correspondence to Arthur L. Beaudet, Henry and Emma Meyer Professor and Chair, Dept. of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Rm. T619, Houston, TX 77030. Phone: 713-798-4795; Fax: 713-798-7773; E-mail: abeaudet@bcm.tmc.edu

Because of the major potential for genetic manipulation in the mouse (10), a variety of strategies including transgenic overexpression, inactivating mutations induced by homologous recombination, and blocking mAbs have been used to study the relationships between leukocyte and endothelial CAMs and other aspects of the inflammatory process associated with atherosclerosis as reviewed elsewhere (11). Antibodies to α_4 -integrin and ICAM-1 have been used in apolipoprotein (apo) E^{-/-} (deficient) mice to demonstrate reduction in recruitment of monocytes to atherosclerotic plaques (12). A blocking antibody to CD40 ligand decreased atherosclerosis in low density lipoprotein receptor (LDLR)^{-/-} mice fed a high-cholesterol diet, with a demonstrated effect on both macrophages and lymphocytes (13). Osteopetrotic mice lacking macrophage colony-stimulating factor were protected against atherosclerosis under a variety of conditions (14, 15). In apo E^{-/-} mice fed a high-fat diet, deficiency for the monocyte chemoattractant protein 1 receptor, CCR2, reduced lesions (16).

Various studies have directly assessed mice lacking expression of one or more CAMs for the effect on atherosclerosis. In a study using C57BL/6 mice fed a high-fat diet, a 50–75% reduction in atherosclerotic fatty streaks was found in mice deficient for ICAM-1, P-selectin, or CD18 (17). In studies of mice deficient for P-selectin or both P- and E-selectin conducted with LDLR^{-/-} mice, a modest effect was seen in male but not female mice lacking P-selectin, whereas a more substantial reduction in lesions was seen in the P- and E-selectin double-deficient mice (18, 19). The P- and E-selectin double-mutant mice develop inflammatory skin disease, which might influence lesion development (20, 21), and the studies of C57BL/6 and LDLR^{-/-} mice involved use of a diet high in cholesterol and cholic acid, the latter being an abnormal supplement to the diet that can itself induce a chronic inflammatory state (10). In a study of leukocyte rolling in the carotid arteries of apo E^{-/-} mice fed a high-fat diet, blocking mAbs to P-selectin or P-selectin ligand 1 decreased mononuclear cell attachment and rolling, whereas blocking antibodies to α_4 -integrin or VCAM-1 increased rolling velocities (22).

We wished to study the effect of genetic deficiency for three individual CAMs (P-selectin, ICAM-1, and E-selectin) in the apo E^{-/-} mouse model, in which mice develop spontaneous lesions in the arterial vasculature with advanced lesions morphologically similar to those seen in humans when fed a regular chow, high-fat, or high-cholesterol diet (23–25). Even with a mouse chow diet low in fat and cholesterol, apo E^{-/-} mice develop spontaneous atherosclerosis including fibroproliferative lesions (24–27) similar to those seen in humans. In the apo E^{-/-} mice on normal chow, we observed substantial reduction in lesions with P-selectin or ICAM-1 deficiency and marginal effects with E-selectin deficiency.

Materials and Methods

Animals and Diet. The ICAM-1 (28), P-selectin (29), and E-selectin (20) mice were generated in our laboratory and were backcrossed onto a C57BL/6 background a minimum of six gen-

erations (N6). Apo E^{-/-} mice (23) were obtained from The Jackson Laboratory and were also backcrossed to C57BL/6 (N6). Mice of the genotype apo E^{-/-}-CAM^{-/-} were generated by matings between the two mutant mouse strains and their progeny to produce three strains of mice double mutant for apo E and ICAM-1, P-selectin, or E-selectin. See Fig. 1 for the breeding scheme used to generate the study mice. One double mutant from each group (apo E^{-/-}-CAM^{-/-}) was crossed back to apo E^{-/-} mice (apo E^{-/-}-CAM^{+/+}) to generate apo E^{-/-}-CAM^{+/-} mice. The progeny of these mice were used in the study. All mice in each adhesion molecule arm of the study were descendants of the same apo E^{-/-}-CAM^{-/-} and apo E^{-/-} grandparental mice. The mice were fed standard mouse chow (Ralston Purina 5001) containing 6% fat and 0.0275% cholesterol from weaning until 20 wk of age. Mice were then killed for lesion analysis. Animals were housed in clean facilities with sentinel animals that consistently tested negative for common viral pathogens. Food and water were provided ad libitum, and an alternating 12-h light–dark cycle was maintained.

Cholesterol Determination. Blood was collected from the retro-orbital venous plexus of anesthetized mice after fasting overnight (16–18 h). Total plasma cholesterol was determined using an enzymatic assay (cholesterol kit 352-20; Sigma Diagnostics) according to the manufacturer's instructions. Cholesterol was separated into very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL)/LDL, and high density lipoprotein (HDL) fractions, subjecting 0.1 ml of plasma to fast performance liquid chromatography gel filtration on two Superose 6 columns (Pharmacia Biotech Inc.) connected in series as described (30). 40 0.5-ml fractions were collected, and cholesterol in each was determined as above, with fractions 6–25 containing VLDL+IDL+LDL and fractions 27–35 containing HDL.

Quantitation of Lesion Area. At 20 wk of age, the mice were killed and their aortas isolated. Aortas were mounted and lesion areas quantitated as described by a person blinded to the genotypes (31). In brief, the cleaned aortas were cut open longitudinally, pinned onto cardboard, and fixed in formalin. After staining with Oil Red O (Sigma Chemical Co.) and mounting on glass slides, the aortas were scanned at high resolution with a SprintScan 35 slide scanner (Polaroid). Lesion areas were then calculated from the scanned images.

Histopathology. The aortas from three C57BL/6 wild-type, three apo E^{-/-} with no CAM deficiency, three apo E^{-/-}-ICAM-1^{-/-}, and three apo E^{-/-}-P-selectin^{-/-} animals were fixed in formalin, dehydrated through graded alcohol and xylene, and then embedded in paraffin. Serial 5- μ m-thick tissue sections were performed, stained with hematoxylin and eosin, and evaluated microscopically in a blinded fashion for lesions. Representative photomicrographs were taken from each specimen for comparison among the groups.

Statistical Analysis. Statistical analysis of the data generated was conducted with Statview (version 4.5; Abacus Concepts, Inc.) statistical analysis software. Two-way analysis of variance (ANOVA) was used to determine the effects of genotype, gender, and the interaction between genotype and gender. This analysis was completed for each of the independent CAM genes tested.

Results

Production of Study Mice. With the starting mice all being backcrossed (\geq N6) and all study mice for each CAM descended from the same grandparental breeding pair (Fig. 1),

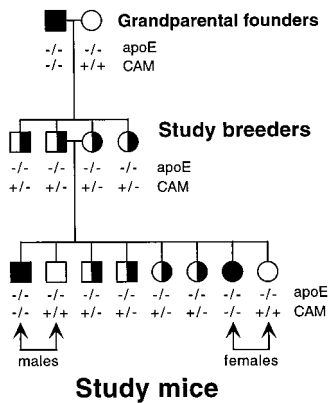


Figure 1. Breeding scheme for generating study animals. All animals in the study were apo E^{-/-}. The animals for each CAM mutation are all descendants of a single founder pair of mice.

the mice used had minimal genetic variation apart from the CAM genotype. Offspring of the breeding pairs, all apo E^{-/-} and +/+, +/-, or -/- for the CAM, were considered as grouped littermates and were kept on the chow diet from weaning until 20 wk of age before the aortas were harvested. There was no difference in appearance or weight (data not shown) of the males or females in any group.

Cholesterol Analysis. Total cholesterol levels were measured in all of the mice for the duration of the study to monitor for possible differences in cholesterol levels (Table I). A Kruskal-Wallis nonparametric ANOVA test indicated no differences in total cholesterol or HDL cholesterol among animals within each experimental group or at any time point during the study. This was true in all groups for 4-wk time points, CAM genotypes, and sexes of mice.

Lesion Analysis. Atherosclerotic lesions are more likely to be observed in specific areas of the aorta. These include the valve cusps, aortic arch, and the abdominal aorta in the region of the renal arteries (Fig. 2). Smaller lesions including fatty streaks were seen throughout the aorta but were more common at arterial branch points. Cross-sections of

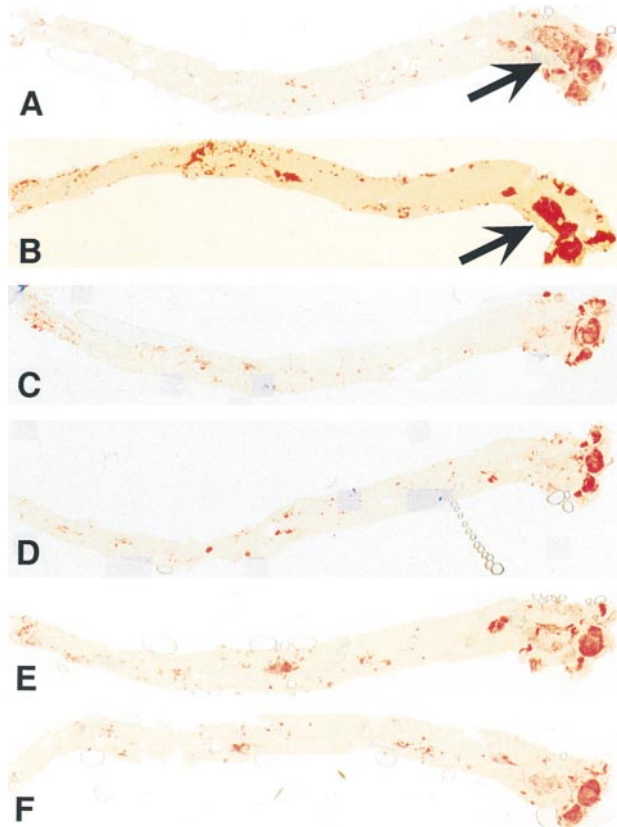


Figure 2. Oil Red O-stained lesions on aortas from apo E^{-/-} mice. (A and B) Representative aortas from P-selectin^{+/+} and ICAM-1^{+/+} males, respectively. Total lesion area on these aortas was 5.81 mm² for A and 6.26 mm² for B. Arrows point to more advanced lesions in the aortic arch. (C and D) Aortas from two P-selectin^{-/-} males with total lesion areas of 3.08 and 2.91 mm², respectively. Note the relative absence of lesions throughout the aorta compared with CAM^{+/+} mice in A and B. (E and F) Aortas from ICAM-1^{-/-} mice with lesion areas of 4.38 and 3.90 mm², respectively. Early lesions are present in the aortic arch.

Table I. Plasma Cholesterol Levels in Study Mice

Genotype	Total cholesterol	HDL cholesterol
	mg/dl ± SD	mg/dl ± SD
apo E ^{-/-} -CAM ^{+/+} (n = 50)	487 ± 152	35 ± 14
apo E ^{-/-} -ICAM-1 ^{-/-} (n = 13)	471 ± 123	41 ± 17
apo E ^{-/-} -P-selectin ^{-/-} (n = 19)	495 ± 134	45 ± 18
apo E ^{-/-} -E-selectin ^{-/-} (n = 18)	503 ± 139	39 ± 15

Mice were fed a mouse chow diet from weaning until 20 wk of age, and plasma cholesterol was determined as described in Materials and Methods at 8, 12, 16, and 20 wk for all mice in the study (n = 100). There was no difference in cholesterol levels (Kruskal-Wallis nonparametric ANOVA) for the time points or sexes within each experimental group (CAM mutation), so they were combined. The amount of HDL cholesterol was determined using fast performance liquid chromatography for five mice in each group with no difference among groups. There was also no significant difference in the cholesterol levels for any experimental group of mice (Kruskal-Wallis).

the most advanced lesions found in C57BL/6 wild-type, apo E^{-/-} with no CAM deficiency, apo E^{-/-}-ICAM-1^{-/-}, and apo E^{-/-}-P-selectin^{-/-} mice are shown in Fig. 3. The calcification seen in the advanced lesions of apo E^{-/-} mice was not observed in any of the aortas analyzed from ICAM-1 or P-selectin null mice. The most advanced lesions seen in the P-selectin^{-/-} mice contained foam cells within expanded intima.

There was very little size variation among the aortas due to the consistent size of the mice. The area for atherosclerotic lesions was determined, comparing the effect deficiency for ICAM-1, P-selectin, and E-selectin in apo E^{-/-} mice, with each gene defining an independent experimental group of animals. Two-way ANOVA showed no significant interaction effect between genotype and gender (ICAM-1, P = 0.81; P-selectin, P = 0.33; and E-selectin, P = 0.55); therefore, male and female lesion area data were combined for calculation of statistical significance. As shown in Fig. 4, mice homozygous null for the ICAM-1 mutation had significantly less lesion formation than their ICAM-1^{+/+} littermates (4.08 ± 0.70 mm² for -/- males vs. 5.87 ± 0.66 mm² for

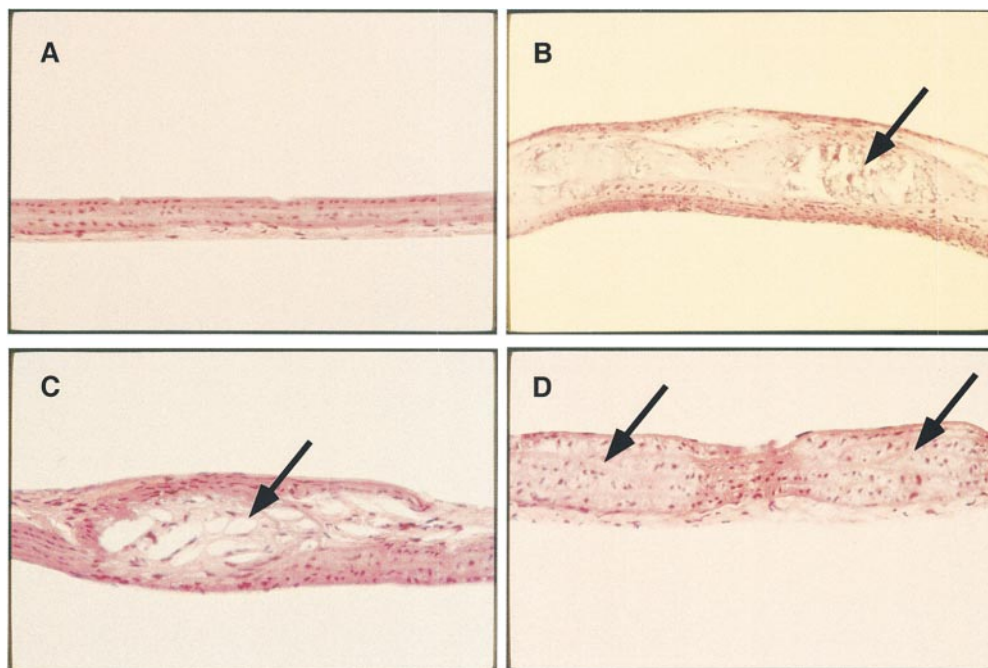


Figure 3. Histopathologic comparison of aortas from study animals. Sections shown are typical of the most advanced lesions found in animals of each genotype. (A) C57BL/6 wild-type murine aorta with intact intimal (top), unremarkable media (middle), and adventitial (bottom) layers with no evidence of atheromatous lesion. (B) Aorta from apo E^{-/-} mouse with no CAM deficiency showing well formed atheromatous plaque characterized by foam cells, cell debris, cholesterol clefts, and calcification (arrow) within the expanded intima. Lesions exhibiting the calcification seen only in very advanced lesions were only found in apo E^{-/-} mice with no CAM deficiency. (C) Aorta from an apo E^{-/-}ICAM-1^{-/-} animal with lesion composed of foam cells, cholesterol clefts, and extracellular lipid in expanded intima (arrow). (D) Aorta from apo E^{-/-} P-selectin^{-/-} animal with less

developed atherosclerotic lesions (arrows) composed of frequent foam cells within expanded intima. Note relatively normal aortic segment between atheromatous lesions. (All hematoxylin and eosin stain with original magnification of 200).

+/+ males, and $3.95 \pm 0.65 \text{ mm}^2$ for -/- females vs. $5.59 \pm 1.13 \text{ mm}^2$ for +/+ females, combined $P < 0.001$). Also shown in Fig. 4 is an even greater reduction of lesion area in P-selectin^{-/-} mice ($3.06 \pm 1.04 \text{ mm}^2$ for -/- males vs. $5.09 \pm 1.22 \text{ mm}^2$ for +/+ males, and $2.85 \pm 1.26 \text{ mm}^2$ for -/- females compared with $5.60 \pm 1.19 \text{ mm}^2$ for +/+ females, combined $P < 0.001$). Most of the aortas from P-selectin^{-/-} mice were remarkably free of lesions except for the valve cusps. The reduction in lesion

area for E-selectin null mice, although less than that seen for ICAM-1 or P-selectin, was still significant ($4.54 \pm 2.14 \text{ mm}^2$ for -/- males vs. $5.92 \pm 0.63 \text{ mm}^2$ for +/+ males, and $4.38 \pm 0.85 \text{ mm}^2$ for -/- females vs. $5.92 \pm 1.44 \text{ mm}^2$ for +/+ females, combined $P < 0.01$). None of the mice heterozygous for CAM had differences in lesion areas compared with apo E^{-/-} CAM^{+/+} mice (data not shown). The lesion area data collected follows a normal distribution, with significant protection from the development of atherosclerosis observed in animals with null mutations in each independent adhesion molecule.

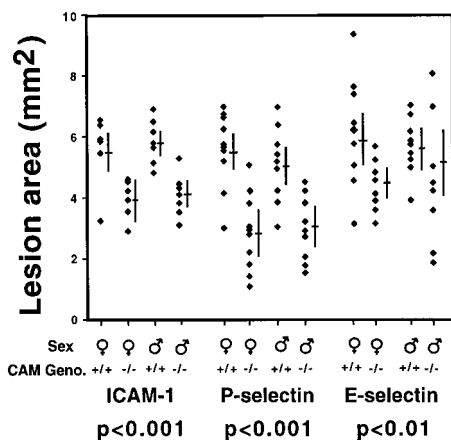


Figure 4. Effect of CAM deficiency on lesion area in apo E^{-/-} mice. Each data point represents total lesion area (mm²) in an animal's aorta between the aortic valve and the iliac branch for the sex and genotype shown. Error bars represent mean \pm SD. Significant reduction in lesion area was seen in the animals of each experimental group (CAM mutation) with the P values shown. Along with the rigid control of genetics and husbandry, two-way ANOVA indicated that the protection seen was due to genotype, with no significant contribution from gender.

Discussion

As reviewed in the Introduction, extensive studies have demonstrated increased expression of leukocyte and endothelial CAMs in atherosclerotic lesions, and genetic manipulation has been used extensively in the mouse to study the pathogenesis of atherosclerosis. In previous studies using a high-fat diet containing cholic acid, individual CAM deficiencies in C57BL/6 mice (17) and P-selectin or P- and E-selectin deficiency in LDLR^{-/-} mice (18, 19), reduction in lesion formation was observed. The studies presented here demonstrate a reduction in atherosclerotic lesions using a more normal low-fat, low-cholesterol mouse chow diet in the apo E^{-/-} mouse model. Importantly, the mice were healthy, closely matched for genetic background and husbandry, and showed no differences in plasma cholesterol. The ICAM-1^{-/-} mice demonstrated a 30% reduction in lesions, and the P-selectin^{-/-} mice demonstrated a 45% reduction in lesions at 20 wk of age; the differences were highly statistically significant for CAM^{-/-} compared with

CAM^{+/+} mice. Lesion reduction in E-selectin^{-/-} mice was not as great, with 24% reduction in lesion area. Histopathological sections show that although foam cells are present in P-selectin null mice and extracellular lipids and cholesterol clefts occur in ICAM-1 null mice, the calcification seen in the very advanced lesions of CAM^{+/+} mice were not found in any of the mice lacking ICAM-1 or P-selectin. Studies of this type can be carried out under many different circumstances, including transgenic expression of lipoprotein(a) or cholesteryl ester transfer protein, various dietary conditions, increased homocysteine levels, and genetic deficiency for various CAMs and other inflammatory molecules. The studies reported here demonstrate that deficiency of P-selectin, ICAM-1, or E-selectin provides substantial reduction in lesions in apo E^{-/-} mice fed a normal chow (low-fat) diet.

There is extensive evidence that monocytes play a pivotal role in the pathogenesis of atherosclerosis (2, 3, 14, 15). Ligands for the three adhesion molecules examined in this study are expressed on the surfaces of monocytes, and their expression is upregulated upon monocyte activation (32). It can be argued that leukocyte and endothelial CAMs play a pivotal role in the pathogenesis of atherosclerosis, and that the effects of many risk factors might be mediated through effects on CAMs. Multiple reports demonstrate that cigarette smoking promotes leukocyte and endothelial adhesion reactions (33–35). There is also evidence to suggest that hyperglycemia and diabetes mellitus might increase the expression of leukocyte and/or endothelial CAMs (36–38), and shear stress selectively upregulates expression of ICAM-1 (39). Modified LDL can increase the expression of CAM (40, 41), and the antiatherogenic effect of probucol may be mediated by reducing the expression of VCAM-1, P-selectin, and other inflammatory mediators (42). There is a positive association of soluble CAMs with carotid atherosclerosis (43). Genetic polymorphisms in selectins may influence the risk of atherosclerosis in humans (44, 45), and two reports support the association of a serine→arginine mutation at codon 128 of E-selectin with coronary artery disease (46, 47). There is also a suggestion that lipoprotein Lp(a) may mediate its proatherogenic effect through upregulation of VCAM-1 and E-selectin (48). It remains to be determined if strategies to reduce the expression or adhesion of leukocyte and endothelial CAMs can be used to achieve protection against atherosclerosis in a clinical setting.

We wish to acknowledge Dr. Klaus Ley and E. O'Brien Smith, Ph.D., biostatistician of the Children's Nutritional Research Center, Baylor College of Medicine, for helpful discussions and critical review of the manuscript. Tanya Allen, Martin Idunoba, and Felton Nails provided technical assistance with histopathology.

A.L. Beaudet was an Investigator with the Howard Hughes Medical Institute during the time that most of this work was performed. This work was also supported by National Institutes of Health grants HL 51586 (to L. Chan) and AI 32117 (to A.L. Beaudet).

Submitted: 3 May 1999
 Revised: 26 August 1999
 Accepted: 18 October 1999

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